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Note

High-performance liquid chromatographic separation of glucose esters and quinic acid esters of hydroxycinnamic acids

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Glucose esters and quinic acid esters of hydroxycinnamic acids, which are widespread in higher plants, are difficult to separate by present methods[1]. Polyamide column chromatography represents an approach to this problem and enables group analyses of these two ester types[2]; however, separation of the individual derivatives requires re-chromatography on thin layers. Thus, the resolution of complex mixtures of these esters is time-consuming and quantitation is difficult.

It has been shown that reversed-phase high-performance liquid chromatography (HPLC) is applicable for the analysis of free hydroxycinnamic acids[3,4]. There have been only a few studies on the separation of simple mixtures of their conjugates by HPLC. Court[5] has reported the HPLC separation of isomers of chlorogenic acid, and Strack and Klug[6] have similarly resolved sinapoylglucose in a mixture of sinapic acid esters. Molderez *et al.*[7] separated glucose and quinic acid esters of *p*-coumaric and caffeic acid on LiChrosorb RP-8 and reported that prepurification and a group separation step were needed prior to the application of HPLC. Ong and Nagel[8] developed an HPLC technique to separate and quantify hydroxycinnamic acid–tartaric acid esters.

This paper describes the resolution of a complex mixture of 10 hydroxycinnamic acid conjugates from tissues of *Spirodela polyrrhiza* (duckweed) without any pretreatment of the crude extracts.

EXPERIMENTAL

Alcoholic extraction of fresh material of *Spirodela polyrrhiza* was done as described previously[9]. Without any pretreatment the crude extract was applied to HPLC. Besides the naturally occurring hydroxycinnamic acid conjugates in *Spirodela*[2], glucose esters of *p*-coumaric acid and caffeic acid were obtained by administration of free *p*-coumaric acid to the intact plant[1] (10 mg/100 ml culture medium for 24 h). Isomers of *p*-coumaroyl- and caffeoylquinic esters were produced by a method described by Sondheimer[10].

The liquid chromatograph used and the chromatographic columns were described in a previous communication[9]. Peak identification was achieved by applying isolated known compounds. HPLC separation was accomplished by gradient elution: in 25 min linear from solvent A (water–methanol–acetic acid, 92.5:5:2.5) to

10% B (water–methanol, 5:95) in A + B. Alternatively acetic acid was replaced by a buffer system (citric acid, 0.1 *M* at defined pH values) in A and B. The flow-rate was 2 ml/min, detection was at 312 nm and the sample size was 25 μl.

Calculations were done with an Autolab System I computing integrator (Spectra-Physics, Santa Clara, Calif., U.S.A.).

RESULTS AND DISCUSSION

The hydroxycinnamic acid conjugates, which were separated with HPLC, are listed in Table I. Fig. 1 shows (a) the resolution of a crude extract of *Spirodela* plants which were incubated with free *p*-coumaric acid (see Experimental) and isomers of *p*-coumaroyl- and caffeoylquinic esters which were added, and (b) the resolution of the quinic acid isomers. As can be seen, the peak pattern allows quantitation of each individual compound.

TABLE I

RELATIVE RETENTIONS (α) OF HYDROXYCINNAMIC ACID CONJUGATES ON LICHROSORB RP-8 USING A WATER–METHANOL GRADIENT

Peak No.	*Compound*	α
1	5-Caffeolquinic acid	1.37
2	1-Caffeoylglucose	1.34
3	5-*p*-Coumaroylquinic acid	1.47
4	4-Caffeoylquinic acid	1.10
5	1-*p*-Coumaroylglucose	1.05
6	3-Caffeoylquinic acid	1.39
7	1-Feruloylglucose	1.11
8	4-*p*-Coumaroylquinic acid	1.06
9	1-Sinapoylglucose	1.22
10	3-*p*-Coumaroylquinic acid	

To determine whether free hydroxycinnamic acids would elute in the retention range of their conjugates, thus interfering with the resolution, we added each of the hydroxycinnamic acids to the *Spirodela* extract. It was observed that these compounds elute after the *Spirodela* constituents. Wulf and Nagel[3] and Ong and Nagel[8] observed a shorter retention time of caffeic acid relative to its 3-quinic acid derivative (chlorogenic acid).

Our results are in agreement with those of Murphy and Stutte[4]. The isocratic system used by Wulf and Nagel[3] for the separation of free hydroxycinnamic acids could not be duplicated in our laboratory. On RP-18, we achieved baseline separation of the four hydroxycinnamic acids using a linear gradient from water to 25% methanol (5% acetic acid in each) in 25 min (flow-rate 2.0 ml/min).

Comparing our results with those of Court[5], we find a difference in the elution sequence of the caffeoylquinic acid esters. In Court's system, the 4-isomer elutes after the 3-isomer, whereas in our profile the 3-isomer is the last of the caffeoylquinic ester isomers to be eluted (see peaks 1, 4 and 6 in Fig. 1).

We found that the content of acetic acid in the elution mixture is very important in the separation of hydroxycinnamic acid conjugates. Slight changes in

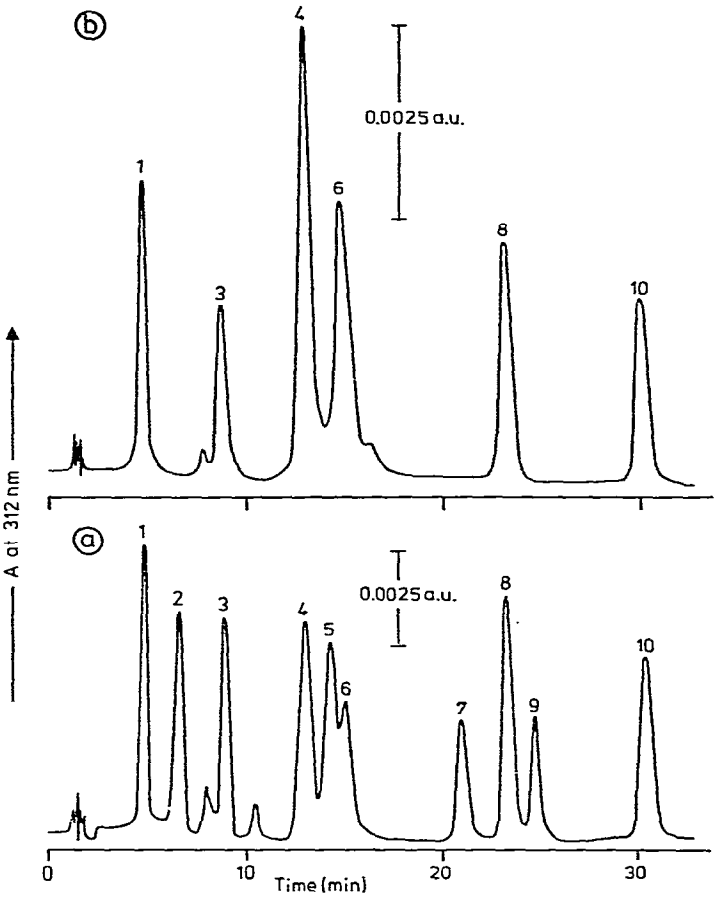

Fig. 1. The separation of hydroxycinnamic acid conjugates on LiChrosorb RP-8 using a water–methanol gradient. (a) The resolution of a crude extract of *Spirodela* plants which were incubated with free *p*-coumaric acid. Isomers of *p*-coumaroyl- and caffeoylquinic acid esters were added to the extract. (b) The resolution of isomers of *p*-coumaroyl- and caffeoylquinic acid esters. For peak identification, see Table I.

concentration (±1%) resulted in marked changes in the retention times and the selectivity. This effect can be used to optimize the separation. We obtained poor resolution using 5% acetic acid, and acetic acid concentrations between 1.5 and 2.5% gave the best resolution pattern.

Ong and Nagel[8] reported a pH sensitivity on the elution of hydroxycinnamic acid–tartaric acid esters and proposed to use this effect to optimize HPLC separation. We examined this pH effect on the isomers of caffeoylquinic acid (1, 4 and 6 in Table I) with a citric acid buffer to find if better separation can be obtained and if the reproducibility is equal to that obtained with 2.5% acetic acid in the solvent mixture.

The influence of different pH values on the capacity factors (k') of the isomers of caffeoylquinic acid is shown in Fig. 2. Whereas little effect was observed on the elution of 5-caffeoylquinic acid, there was a marked influence on 3- and 4-caffeoyl-

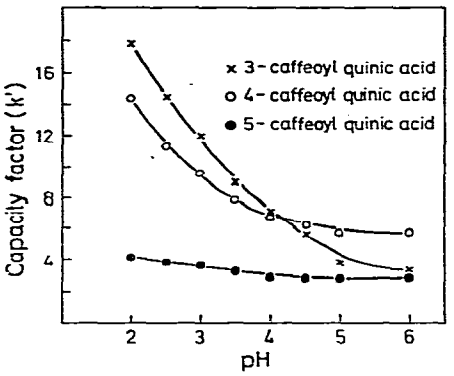

Fig. 2. The effect of pH (0.1 *M* citric acid buffer) on the capacity factors (k') of isomers of caffeoylquinic acid. Each point represents the mean of three determinations.

quinic acid. By shifting the k' values of these two compounds by changing the pH of the solvent mixture, the relative retention (α) for these isomers can be optimized; at pH 5.0 the α-value is 1.48 whereas with 2.5% acetic acid α is 1.16. In addition, the pH of the applied buffer affects the elution sequence (see Fig. 2), which could explain the difference from the results of other authors[3,5,8] (see above).

The reproducibility attained in quantitative analyses with the two elution systems is shown in Table II. The coefficients of variation ($n = 6$) demonstrate that better reproducibility can be obtained for both k' values and peak areas (integrator units) when 2.5% acetic acid is used in the solvent mixture for HPLC of caffeoylquinic acid esters.

TABLE II

CAPACITY FACTORS (k'), INTEGRATED PEAK AREAS AND COEFFICIENTS OF VARIATION (C.V.) OF HPLC-SEPARATED ISOMERS OF CAFFEOYLQUINIC ACID OBTAINED WITH 2.5% ACETIC ACID OR CITRIC ACID BUFFER

Compound	*2.5% Acetic acid*				*Buffer (pH 5.0)*			
	*k'**	*C.V. (%)*	*Area**	*C.V. (%)*	*k'**	*C.V. (%)*	*Area**	*C.V. (%)*
5-Caffeoylquinic acid	2.56	0.66	76,909	1.15	2.75	2.43	75,281	4.41
4-Caffeoylquinic acid	6.03	0.66	71,130	0.27	5.55	2.32	72,501	1.21
3-Caffeoylquinic acid	7.03	0.56	126.514	0.17	3.75	2.32	121,376	0.81

* Average of six runs.

In conclusion, this HPLC system appears to be applicable to physiological studies on the metabolism of hydroxycinnamic acid conjugates in *Spirodela* plants. This plant contains a mixture of quinic acid and glucose derivatives, whose separation with classical methods is elaborate and time-consuming. HPLC offers an efficient and dependable method for investigations on phenylpropanoid metabolism.

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